

A universal method to produce *in vitro* transcripts with homogeneous 3′ ends

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ABSTRACT

A method is described that allows a general drawback of *in vitro* transcription assays to be overcome: RNA polymerases tend to add extra nucleotides to the RNA 3′ end that are not encoded in the linearized DNA template. Furthermore, these polymerases show a considerable rate of premature termination close to the RNA's 3′ end. These features lead to a decreased yield of full-length transcripts and often make it difficult to determine and isolate the correctly transcribed full-length RNA. The hammerhead ribozyme is frequently used in *cis* to cleave off these extra nucleotides. However, the upstream sequence requirements of this ribozyme restrict its general usability. In contrast, the hepatitis delta virus ribozyme has no such requirements and can therefore be applied to any RNA sequence in *cis*. Due to the catalytic activity of the ribozyme, the desired transcript is released as an RNA molecule with a homogeneous 3′ end. The resulting 2′,3′-cyclophosphate group of the released RNA can be easily and efficiently removed by T4 polynucleotide kinase treatment. The presented method can be applied for virtually any sequence to be transcribed and is therefore superior to other ribozyme strategies, suggesting possible applications in every field where transcripts with homogeneous 3′ ends are required.

INTRODUCTION

The *in vitro* synthesis of RNA molecules is a widely used technique in molecular biology laboratories (1–3). Usually, the gene of interest is inserted into special vector plasmids carrying a phage promoter (T7, T3, SP6) upstream of the multiple cloning site. These vectors are then linearized by restriction enzymes and used for run-off transcription with the appropriate RNA polymerase. Another frequently used type of run-off transcription template consists of PCR products with a promoter sequence introduced by one of the PCR primers.

While this technique is very useful and the quality of the resulting transcripts sufficient for many applications, it does not meet the requirements for experiments where homogeneity of the transcripts is needed. Since RNA as well as DNA polymerases (such as *Taq* polymerase) show a tendency to fall off the template before they reach the very end of the DNA (premature termination) or add non-encoded nucleotides to the 3′ end of the nascent nucleic acid, the resulting transcripts usually show highly heterogeneous 3′ ends (3–5). This fact often makes it impossible to isolate the correct transcript band from a preparative polyacrylamide gel and leads to a dramatically decreased yield of correctly sized RNA (see Fig. 2, left).

In order to improve the yield of correctly terminated transcripts and to reduce the amount of incomplete or elongated RNAs, previously described methods use hammerhead or hairpin ribozymes as *cis*-cleaving autocatalytic cassettes (6). However, due to specific upstream sequence requirements, the applicability of these cassettes is restricted to certain RNA sequences. To overcome this drawback, we established a method that uses an optimized version of the hepatitis delta virus (HDV) ribozyme to generate homogeneous RNA 3′ ends (7,8). The HDV ribozyme is the only known catalytic RNA domain that has almost no prerequisites on the sequence composition upstream of the cleavage position and is therefore perfectly suited for such a strategy (9,10). In our model system, we used a chimpanzee mitochondrial $tRNA^{Phe}$ that should be produced as a homogeneous *in vitro* transcript.

MATERIALS AND METHODS

Construction of HDV-tRNA constructs

The deletion variant of the HDV ribozyme (ΔHDV, 5′-GGG UCG GCA UGG CAU CUC CAC CUC CUC GCG GUC CGA CCU GGG CUA CUU CGG UAG GCU AAG GGA GAA G-3′, 67 nt) was constructed by PCR (Fig. 1A: construction, 20 cycles of 1 min, 94°C; 1 min, 60°C; 30 s, 72°C) using two primers which overlap at their 3′ terminus for 23 bases (underlined): HDVforward (5′-GGG TCG GCA TGG CAT CTC CAC CTC CTC GCG GTC CGA CCT GGG CTA-3′) and HDVreverse (5′-CTT CTC CCT TAG CCT ACC GAA GTA GCC CAG GTC GGA CCG CGA GGA-3′). A second PCR reaction (2 min, 94°C; 40 cycles of 1 min, 94°C; 1 min, 56°C; 30 s, 72°C) was performed to amplify the construct of the first PCR, introducing an elongated downstream primer (Fig. 1A, amplification): HDVamp/forward (5′-GGG TCG GCA TGG CAT CTC CAC-3′) and HDVext/reverse (5′-AAA CGA CGG CCA GTG CCA AGC TTC TCC CTT AG-3′). This elongation does not influence the reactivity of the ribozyme, but makes it easier to distinguish between the bands of the released tRNA transcript of interest (70 nt) and the HDV ribozyme (87 nt) on an acrylamide gel.

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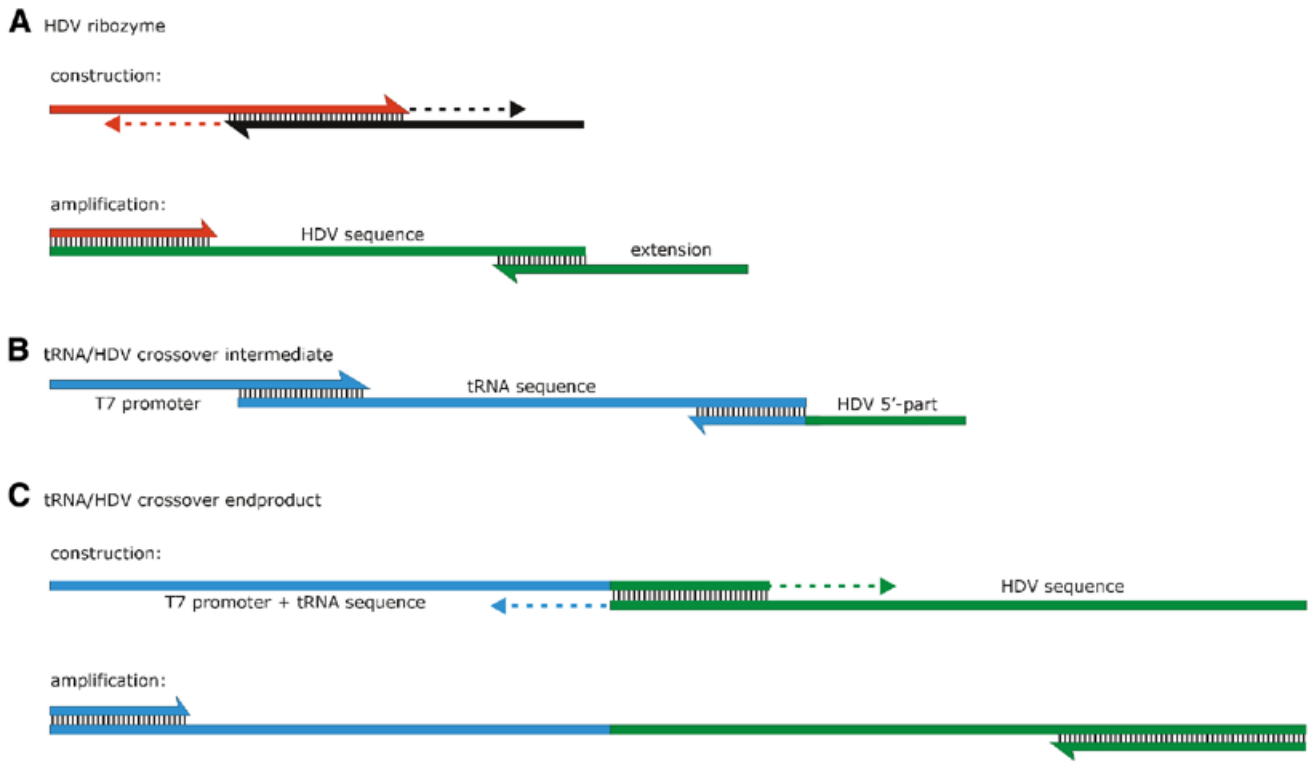

Figure 1. Scheme showing the construction of PCR products consisting of an RNA sequence of interest attached to the HDV ribozyme sequence. (**A**) Construction of the HDV ribozyme sequence starting with an overlapping primer pair. (**B**) Amplification of the RNA sequence of interest as a PCR product carrying the 5′ part of the ribozyme sequence. (**C**) HDV PCR product A is used together with PCR product B in order to create the full-length PCR product consisting of an upstream T7 promoter, the sequence of interest, and the adjacent HDV ribozyme sequence. This DNA molecule is subsequently used for *in vitro* transcription. Fragment length is proportional to corresponding sequences. Size and sequences are given under Materials and Methods.

PCR amplification of the tRNA substrate (2 min, 94°C; 30 cycles: 1 min, 94°C; 1 min, 45°C; 1 min, 72°C) was performed using the following primers (Fig. 1B; tRNA/HDV crossover intermediate): tRNA Phe-T7 (5′-GGA GAT CTA ATA CGA CTC ACT ATA GTT TAT GTA GCT TAC C-3′) and tRNA Phe HDV reverse (5′-TGG AGA TGC CAT GCC GAC CCT GTT TAT GGG GTG-3′). The resulting PCR product had a length of 115 bp.

The final tRNA/HDV crossover construct (182 bp) was created using an overlap extension PCR assay (11,12) (Fig. 1C; tRNA/HDV crossover end product). PCR products A and B were incubated in a PCR assay without addition of primers (4 min, 94°C; 10 cycles: 1 min, 94°C; 2 min, 40°C; 45 s, 72°C). After addition of the primer pair (tRNA Phe-T7 and HDVext/reverse) a standard PCR was performed to amplify the final construct consisting of T7 promoter, $tRNA^{Phe}$ gene and HDV ribozyme sequence.

For the construction of transcription templates containing A, G or T residues immediately upstream of the HDV sequence, the procedure steps B and C (Fig. 1) were repeated using the appropriate tRNA Phe HDV reverse primers.

Transcription

Transcription using T7 RNA polymerase (New England Biolabs, Schwalbach, Germany) was carried out in the presence of [α-^{33}P]UTP according to the supplier's instructions. Transcripts were separated on a 10% polyacrylamide gel containing 8 M urea and visualized by autoradiography. The band corresponding to the tRNA released by the HDV ribozyme self-cleavage reaction was cut out with a sterile blade. tRNAs were eluted by incubation in 500 mM ammonium acetate, pH 5.7, 0.1 mM EDTA, 0.1% SDS at 4°C overnight and ethanol precipitated (13).

Dephosphorylation of RNA 3′ ends

Radioactively labeled transcripts carrying a 2′,3′ cyclic phosphate at the 3′ end (as a consequence of the self-cleavage reaction of the downstream HDV ribozyme) were dephosphorylated using either of the following methods. (i) Up to 50 pmol RNA were incubated with 6 U T4 polynucleotide kinase (New England Biolabs) in 100 mM Tris–HCl pH 6.5, 100 mM magnesium acetate, 5 mM β-mercaptoethanol in a final volume of 50 µl for 6 h at 37°C (14). (ii) Transcripts were incubated with 0.1 mM ATP, 100 mM imidazole–HCl pH 6.0, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 20 µg/ml BSA and 1 U T4 polynucleotide kinase (New England Biolabs) per 100 pmol RNA in a final volume of 50 µl for 6 h at 37°C (15). (iii) Up to 300 pmol tRNA were incubated in 100 mM morpholinoethanesulfonate-NaOH pH 5.5, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 300 mM NaCl and 10 U T4 polynucleotide kinase (New England Biolabs) in a final volume of 20 µl for 6 h at 37°C (16).

All assays were desalted on microspin G25 columns according to the manufacturer (Amersham Pharmacia Biotech, Piscataway, USA), subjected to phenol/chloroform extraction, ethanol precipitated and dissolved in water.

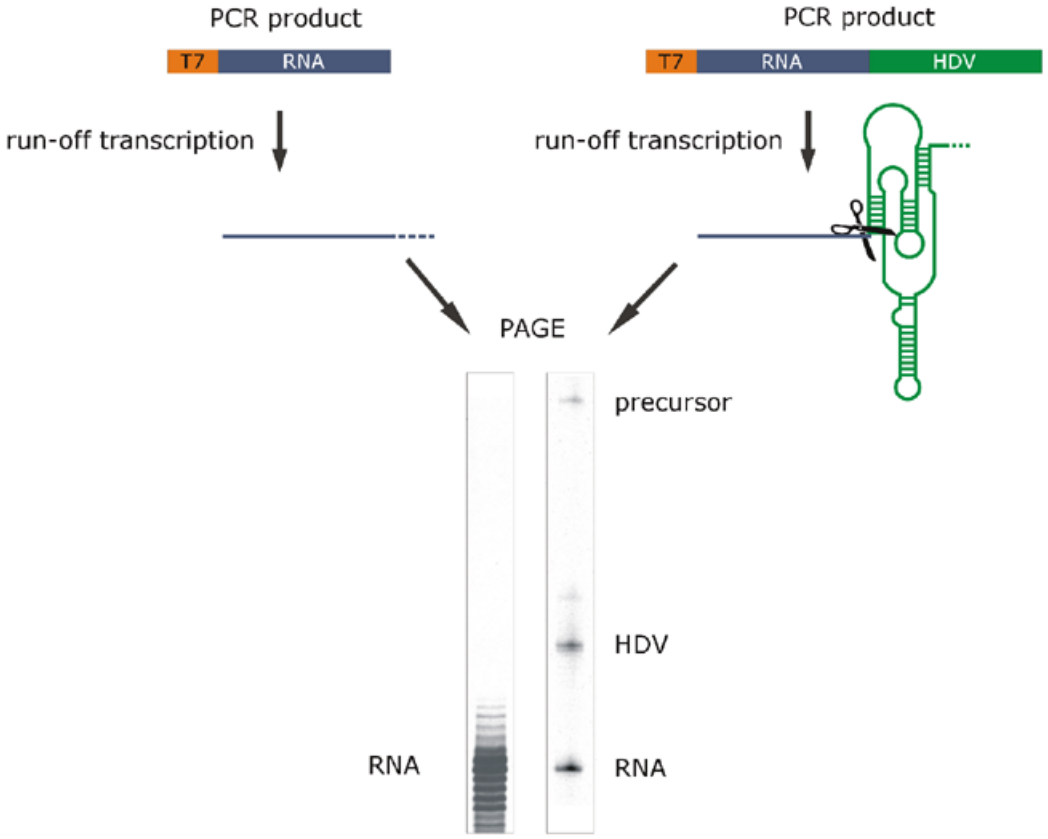

Figure 2. Comparison of the conventional run-off transcription and the ribozyme mediated generation of homogeneous transcripts on a denaturing 10% polyacrylamide gel. While the run-off transcription leads to a set of products of different length, the ribozyme technique leads to one single band corresponding to the transcript of the expected size (RNA) and the released ribozyme (HDV). No premature termination products or transcripts carrying non-encoded extra nucleotides are detectable.

The efficiency of dephosphorylation was controlled by analyzing 1 pmol RNA on a 10% polyacrylamide gel containing 8 M urea. The removal of the 2′,3′ cyclic phosphate leads to a reduction in the net charge of the RNA and results therefore in a decrease in mobility which can be easily observed (see Fig. 4) (16).

3′-Sequence analysis

Isolated tRNAs were ligated to an *Eco*RI linearized pUC18 restriction fragment using T4 RNA ligase. Subsequent cDNA synthesis and PCR amplification were performed as described (17). The PCR products were cloned using a TA cloning kit according to the manufacturer's instructions (Invitrogen, Groningen, The Netherlands). DNA sequencing was carried out on an ABI Prism 377 automated sequencer.

RESULTS AND DISCUSSION

In order to achieve the highest catalytic activity of the HDV ribozyme for an optimal yield of the desired transcript, a slightly modified version containing a shortened form of stem 4 was used (7,8). To generate the transcription template encoding the desired RNA sequence followed by the HDV ribozyme, an overlap extension PCR technique was applied (Fig. 1). The resulting tRNA/HDV PCR product was used for run-off transcription and the obtained RNA was separated on a 10% polyacrylamide gel and visualized by autoradiography (Fig. 2, right). The figure shows three major bands appearing in the gel, representing the precursor transcript, and the two cleavage products consisting of the released RNA molecule and the free HDV ribozyme. This result indicates that the ribozyme is active during transcription, leading to a highly efficient release of the RNA of interest, a fact that renders additional time-consuming incubations for the cleavage reaction unnecessary. Furthermore, compared with the usual run-off transcription technique which leads to heterogeneous 3′ termini (Fig. 2, left), the ribozyme reaction leads to a released RNA of interest that has a defined length, as indicated by the main product band in the autoradiography, while side products are represented only by very faint bands (Fig. 2, right). Sequence analysis of the RNA 3′ end according to Hetzer and Mueller (17) confirmed that the released RNA had the correct size and carried the expected 3′ terminus, whereas the amount of side products was below the detection level.

Therefore, the use of the HDV ribozyme leads not only to a facilitated isolation of correctly sized transcripts, but also to an increase in yield, since nearly no side products with incorrect 3′ ends appear.

While there is no virtual requirement for a specific sequence upstream of the ribozyme, several authors report on base preferences 5′ of the cleavage position by the HDV domain (9,18). However, when we replaced the base upstream of the cleavage

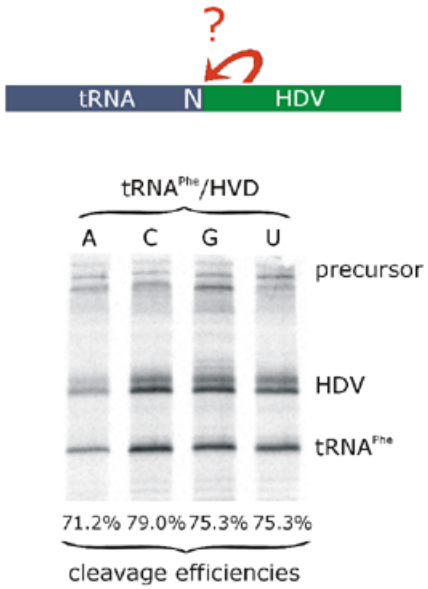

Figure 3. Influence of the nucleotide immediately upstream of the cleavage position. The calculated cleavage efficiencies as the ratio between the signal intensities of the released $tRNA^{Phe}$ and the remaining precursor molecules show that all four nucleotides are tolerated by the ribozyme.

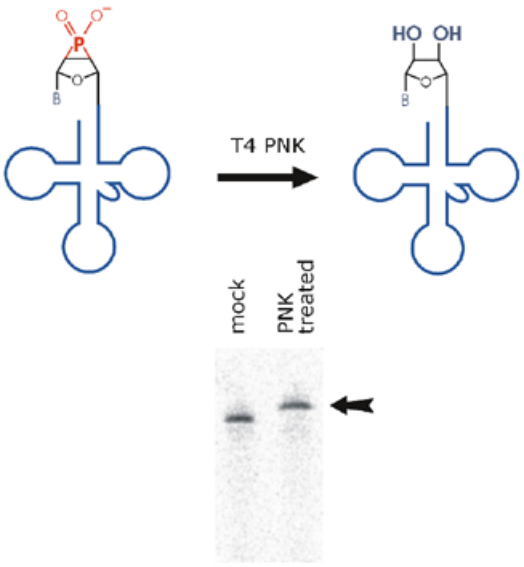

Figure 4. Removal of the terminal 2′,3′ cyclic phosphate group of the released tRNA resulting from the HDV ribozyme cleavage reaction. Treatment with T4 polynucleotide kinase (T4 PNK) leads to the removal of the phosphate group and therefore to a reduced net charge of the RNA. This can be observed by a reduced electrophoretic mobility on a denaturing 10% polyacrylamide gel in comparison with the untreated control (mock incubation without T4 PNK).

position and compared the cleavage efficiency of all four variants as a ratio between the signal intensities of the released products and the remaining precursor transcript on a denaturing 10% polyacrylamide gel, we observed no dramatic differences (Fig. 3): the naturally occurring C residue led only to a marginally increased cleavage efficiency (79.0%) in comparison with A (71.2%), U (75.3%) or G (75.3%). Furthermore, alternative upstream tRNAs that were tested in our laboratory showed consistently similar cleavage efficiencies as the example presented in this study.

Similar to the hammerhead cleavage, the HDV ribozyme reaction leads to a released RNA of interest that carries a 2′,3′ cyclic phosphate group at the 3′ end. Such a 3′ terminus interferes with many functional aspects of RNA molecules. For instance, such a transcript cannot be used for ligation reactions or base incorporation assays. Furthermore, tRNA molecules carrying a 2′,3′ cyclic phosphate group are no longer substrates for aminoacyl tRNA synthetases and can consequently not be used in aminoacylation reactions. Therefore, in order to render these transcripts functional, the interfering cyclic phosphate group has to be removed. In the literature, several techniques are described that use a 3′-phosphatase activity of T4 polynucleotide kinase—an enzyme that is known to add a phosphate group to 5′-hydroxyl ends of both RNA and DNA (14–16,19). In order to find a suitable and convenient method to remove the 2′,3′ cyclic phosphate group, three different protocols with the released transcripts of interest were tested. All methods turned out to work well with the used transcripts and led to a complete removal of the terminal 2′,3′ cyclic phosphate group, a reaction that can be monitored by the reduction of the electrophoretic mobility of the RNA on a denaturing 10% polyacrylamide gel due to the loss of negative charges (Fig. 4).

In order to prove the functionality of this 3′-dephosphorylated tRNA, the isolated transcript was tested for its ability to function as a substrate for CCA addition by ATP(CTP):tRNA nucleotidyltransferase. Almost 100% of the T4 polynucleotide kinase treated tRNA showed CCA incorporation, indicating that the dephosphorylation of the 3′ end led to a functional RNA molecule.

Taken together, these results demonstrate that the utilization of the HDV ribozyme allows the generation of RNAs of defined length carrying functional 3′ termini without the usual side reaction products due to premature termination or incorporation of non-encoded extra nucleotides. Furthermore, the 2′,3′ cyclic phosphate group at the RNA terminus as a result of the ribozyme cleavage reaction does not limit the usefulness of the method, since it can be removed efficiently using the 3′-phosphatase activity of T4 polynucleotide kinase. While the usability of other ribozymes like hammerhead or hairpin is restricted to certain RNA sequences, the HDV ribozyme has no such constraints, but can be applied to virtually any transcription assay where homogeneous RNA 3′ ends are required. Examples for such applications are RNA crystallization and NMR studies. Whereas homogeneous RNA preparations tend to crystallize under several conditions, the use of heterogeneous RNA samples often implies problems due to different conformations in the RNA subpopulations (20). Furthermore, heterogeneous RNA preparations may cause unwanted quenching effects in binding studies using fluorescence labeled RNA (21).

Therefore, this method should be useful for many different biological, biochemical and biophysical applications where homogeneous *in vitro* transcripts are a prerequisite for successful experiments.

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